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Biochemical Systematics of Five Asteroids of the Family Asteriidae Based on Allozyme Variation

Norimasa Matsuoka[1], Kiyoko Fukuda, Kyoko Yoshida,
Miho Sugawara and Megumi Inamori

Department of Biology, Faculty of Science, Hirosaki University, Hirosaki 036, Japan

ABSTRACT—The family Asteriidae of the order Forcipulatida from Japanese waters includes the five common starfish species belonging to the five different genera. They are *Asterias amurensis, Aphelasterias japonica, Distolasterias nipon, Coscinasterias acutispina* and *Plazaster borealis*. The phylogenetic relationship of these five members were investigated by electrophoretic analyses of 15 different enzymes. From the allozyme variation observed in 31 genetic loci, the Nei's genetic distances between species were calculated and the molecular phylogenetic tree for the five species was constructed. The phylogenetic tree indicated the following: (1) The five species are phylogenetically divided into three clusters: (i) *A. amurensis* and *P. borealis*; (ii) *A. japonica* and *D. nipon*; and (iii) *C. acutispina*. (2) *A. amurensis* and *P. borealis* are the most closely related to each other and more recent species which evolved later. (3) *A. japonica* is more closely related to *D. nipon* than to other species. (4) *C. acutispina* is the most distant species of the five members. These electrophoretic results were discussed through the detailed comparison with molecular and non-molecular data, and the differentiation process of five species was speculated.

INTRODUCTION

During the last 10–15 years, the taxonomic, phylogenetic and evolutionary studies have been revitalized by the application of techniques from biochemistry or molecular biology. Protein sequencing, immunological methods, protein electrophoresis, DNA hybridization test and sequence analysis of mitochondrial DNA or ribosomal RNA (DNA) are among the molecular techniques used in evolutionary studies. Of these, enzyme electrophoresis has been most widely used in the field of biochemical systematics [5]. Such molecular studies have made it possible for us to estimate the phylogenetic relationships among taxa and their evolutionary processes quantitatively with common parameters such as enzymes or DNA, and they have been providing much relevant, and in some cases critical, information about phylogenetic relationships in various groups of organisms [10].

One of the present authors (N.M.) has been investigating the phylogeny, taxonomy and evolution within the class Echinoidea (sea-urchins), which is one of the major groups of the phylum Echinodermata, by using the electrophoretic and immunological techniques [13–17, 19, 20]. Another large group of Echinodermata is the class Asteroidea (starfish) and we are also interested in the evolutionary aspect of starfish. The taxonomy and phylogeny of the starfish have been extensively studied by many workers from the morphological and/or paleontological standpoint [1, 2, 4, 9, 28]. However, there are disagreements between asteroid taxonomists, and many unresolved problems concerning the phylogenetic and evolutionary relationships among starfish still remain. For an elucidation of these problems, it would be desirable to actively introduce the molecular approaches which are more analytic and quantitative than the traditional and usual morphological methods into the field of asteroid taxonomy. As already mentioned above, we have been investigating biochemically the phylogenetic relationships among sea-urchins, and found that enzyme electrophoresis is one of the reliable methods in the field of echinoid phylogeny and taxonomy. Therefore, we have an advantage in the biochemical systematic studies of the starfish belonging to echinoderms by enzyme electrophoresis. In the present study, with the background noted above, we have attempted to investigate the phylogeny within the family Asteriidae from the order Forcipulatida by using enzyme electrophoresis.

Five common species were adopted in the present study. They are *Asterias amurensis, Aphelasterias japonica, Distolasterias nipon, Coscinasterias acutispina,* and *Plazaster borealis*. As evident from Figure 1, the former three species have standard five-armed forms, while the latter multi-armed forms. Particularly, *P. borealis* has many arms and shows the clear differentiation between arms and disk. They are common starfish to many zoologists, because *A. amurensis* which is a representative species of the family has been widely used in the embryological, physiological or biochemical study. *C. acutispina* is widely distributed from central Honshu to the Ryukyus, while the other four species are arctic starfish which are commonly found in the seas of northern Japan. Although each of these five species of the family has the characteristic external morphology, it seems to be difficult to establish their phylogenetic relationship and the sequence of the evolutionary divergence by the morphological criteria. Further, there is a little quantitative information available concerning the phylogenetic relationship among these members of the family. In fact, as far as we are aware, there are

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[1] To whom reprint requests should be addressed.

only a few reports: the immunological studies by Kubo [12] and Mochizuki and Hori [22]. Under such situation, further biochemical systematic studies of these species would be desirable and informative.

In this paper, we report on the results of an electrophoretic study designed to clarify the phylogenetic relationship of the five common starfish species of the family Asteriidae from Japanese waters.

MATERIALS AND METHODS

Starfish

The starfish examined in this study were five species from the family Asteriidae of the order Forcipulatida: *Asterias amurensis* Lütken, *Aphelasterias japonica* (Bell), *Distolasterias nipon* (Döderlein), *Coscinasterias acutispina* (Stimpson), and *Plazaster borealis* (Uchida) (Fig. 1). *A. amurensis* and *A. japonica* were collected from the coast near the Asamushi Marine Biological Station, Tohoku University, facing Mutsu Bay, Aomori Pref., by snorkelling. *D. nipon* and *P. borealis* were provided by the Fishermen's Cooperative Association of Yokohama-machi, Kamikita-gun, Aomori Pref. They were collected in the breeding ground of scallops in Mutsu Bay by fishermen. *C. acutispina* was provided by the Misaki Marine Biological Station, University of Tokyo. It was collected from the rocky shore near the Station facing Sagami Bay, Kanagawa Pref. The number of individuals examined was 20 for *A. amurensis*, 39 for *A. japonica*, 20 for *D. nipon*, 21 for *C. acutispina*, and 9 for *P. borealis*. After collection, the pyloric ceaca were cut off from these specimens and stored at −80°C until being analyzed.

Electrophoresis

Electrophoresis was performed on 7.5% polyacrylamide gels by the method of Davis [3] as described previously [14]: About 1 g of pyloric caecum was individually homogenized in 2 vols of 20 mM phosphate buffer, pH 7.0, containing 0.1 M KCl and 1 mM EDTA by using a glass homogenizer of the Potter-Elvehjem type in an ice water bath. After centrifugation at 108,800×*g* for 20 min at 4°C, 0.05–0.10 ml of clear supernatant was used for electrophoretic analyses of enzymes. Electrode buffer was 0.38 M glycine-tris buffer, pH 8.3. After electrophoresis, the gels were stained for the following 15 different enzymes: hexose-6-phosphate dehydrogenase (H6PD), malate dehydrogenase (MDH), malic enzyme (ME), nothing dehydrogenase (NDH), octanol dehydrogenase (ODH), sorbitol dehydrogenase (SDH), xanthine dehydrogenase (XDH), glucose-6-phosphate isomerase (GPI), hexokinase (HK), superoxide dismutase (SOD), aspartate aminotransferase (AAT), alkaline phosphatase (ALK), peroxidase (PO), esterase (EST), and leucine amino peptidase (LAP). Stain recipes for these enzymes have been described previously [21].

RESULTS

From the allozyme variation observed in 15 different enzymes, 31 genetic loci were inferred. Figure 2 shows diagramatically allozyme patterns of four enzymes presenting the typical electrophoretic band patterns, which were chosen from among 15 enzymes analyzed in this study. The major features of variation in these enzymes are summarized as follows: ME exhibited a single band of the same mobility

Fig. 1. Five starfish species of the family Asteriidae from Japanese waters. 1=*Asterias amurensis*, 2=*Aphelasterias japonica*, 3=*Distolasterias nipon*, 4=*Coscinasterias acutispina*, 5=*Plazaster borealis*.

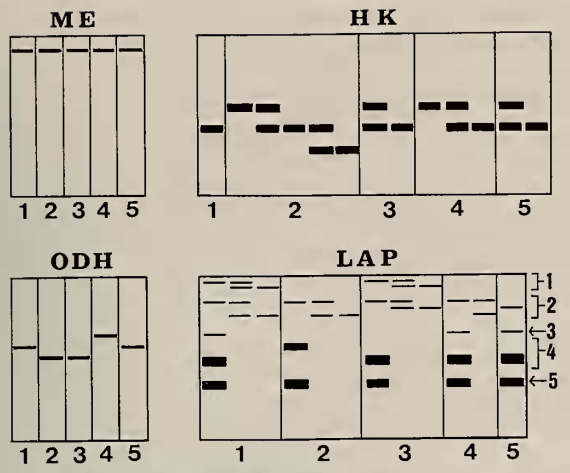

FIG. 2. Electrophoretic band patterns of four typical enzymes in five starfish species of the family Asteriidae. For each enzyme the origin is at the top and the direction of mobility toward the bottom. The number of 1–5 described in the right side of the zymogram of LAP shows the genetic loci of LAP-1 to LAP-5. Genetic loci are numbered downwards from 1, starting with that nearest the origin (i. e., of lowest electrophoretic mobility). The number of 1–5 described in the bottom of each enzyme shows the five starfish species: 1=*Asterias amurensis*, 2=*Aphelasterias japonica*, 3=*Coscinasterias acutispina*, 4=*Distolasterias nipon*, 5=*Plazaster borealis*.

between five species and was monomorphic as well as NDH. ODH also showed a single active band, but varied interspecifically. The similar band pattern was also observed in H6PD and GPI. HK showed extensive polymorphism and exhibited single- and double-banded phenotypes. This variation was interpreted as a diallelic system at a single locus coding for a monomeric protein, with single-banded pattern corresponding to the homozygous state, and double-banded pattern to the heterozygous state. The similar variation was also observed in the following 12 loci: XDH, AAT, MDH-1, PO-2, SOD-3, SOD-4, ALK-1, ALK-4, EST-1, EST-4, LAP-1, and LAP-2. LAP of digestive enzyme was detected as several bands which were grouped into five zones (LAP-1 to LAP-5). LAP-1 and LAP-2 showed single- and double-banded phenotypes as well as HK. The single band of LAP-3 was not scored in two species. Each of LAP-4 and LAP-5 exhibited a single monomorphic band of high enzymatic activity. The multi-banded patterns such as LAP were also observed in EST, ALK and SOD. The LAP activity of the starfish was much stronger than that of various sea-urchin species reported previously at the electrophoretic level [20]. On the other hand, the AMY activity which was scored easily in sea-urchins could not be detected in these starfish species.

The allele frequencies for all loci in the five species are given in Table 1. With respect to the degree of enzyme variation within populations, Table 1 shows that enzymes involved in glucose metabolism (catalysing steps in, or adjacent to, the glycolytic pathway and tricarboxylic acid cycle) were on average less variable than those (e. g., EST or SOD) involved in other reactions, which contain many that are relatively nonspecific with respect to substrate. Table 2 summarizes the extent of genetic variation in five species. The number of alleles per locus was in the range of 1.15–1.38, with a mean of 1.22, the proportion of polymorphic loci (P), in the range of 14.3–38.5%, with a mean of 21.4%, and the expected average heterozygosity per locus (H), in the range of 5.9–16.7%, with a mean of 9.0%. As evident from this table, *D. nipon* showed considerably higher genetic variability than the other four species.

In order to quantify the degree of genetic differentiation among five species, the genetic identity (I) and genetic distance (D) between each species were calculated by the method of Nei [23] from the allele frequencies data in Table 1. Table 3 shows the matrices of I and D values between all pairs of species examined. The highest I value (0.598) was found between *A. amurensis* and *P. borealis*. Figure 3 shows the molecular phylogenetic tree for the five species which was constructed from the Nei's genetic distance matrix of Table 3 by using the unweighted pair-group arithmetic average (UPGMA) clustering method of Sneath and Sokal [27]. The molecular phylogenetic tree indicated the following:

(1) The five species are phylogenetically divided into three large clusters: (i) *A. amurensis* and *P. borealis*; (ii) *A. japonica* and *D. nipon*; and (iii) *C. acutispina*.

(2) Of the five species, *A. amurensis* and *P. borealis* are the most closely related to each other.

(3) *A. japonica* is more closely related to *D. nipon* than to the other three species.

(4) *C. acutispina* is the most distinct species of the five members.

The divergence time (T) of the five species estimated from the genetic distance by the Nei's equation [24] is also given in the phylogenetic tree. The molecular phylogenetic tree with the divergence time provides valuable information with respect to the evolutionary divergence of the five species of the family Asteriidae.

DISCUSSION

Enzyme variation within populations

With respect to the relationship between enzyme function and heterozygosity, Yamazaki [30] showed, using data from various *Drosophila* species, that the substrate-specific enzymes have lower heterozygosity than the nonspecific enzymes. A similar analysis was carried out by Gojobori [7] using data on 20 different proteins (mostly enzymes) from 14 *Drosophila* species, 14 *Anolis* species and 31 other species. As a result, he found that enzymes with various functional constraints tend to have low heterozygosity. These findings are well consistent with our serial electrophoretic studies of echinoderm enzymes. In this study, the glucose metabolizing enzymes (the mean H=6.0%) with functional constraints were less variable than the non-glucose metabolizing enzymes (the mean H=9.9%), and also nonspecific enzymes such as SOD or EST were more highly polymorphic. The similar results have also been obtained in many other echinoderm

TABLE 1. Allele frequencies at various enzyme loci in the five species of the family Asteriidae

Locus	*Aa*	*Aj*	*Ca*	*Dn*	*Pb*
H6PD	b	b	a	b	b
MDH-1	b	b	—	a (0.45)	—
				b (0.55)	
MDH-2	a	c	b	—	a
ME	a	a	a	a	a
NDH	a	a	a	a	a
ODH	b	c	c	a	b
SDH	b	b	b	b	a
XDH	a (0.53)	b	b	c	b
	b (0.47)				
HK	b	a (0.07)	a (0.04)	a (0.47)	a (0.20)
		b (0.76)	b (0.96)	b (0.53)	b (0.80)
		c (0.17)			
PGI	b	b	a	c	—
AAT	b	b	b	a (0.39)	a
				b (0.61)	
SOD-1	a	c	b	—	a
SOD-2	—	a	b	b	a
SOD-3	b	a	—	a (0.73)	—
				c (0.27)	
SOD-4	a	a	c	a (0.75)	a
				b (0.25)	
PO-1	b	a	c	—	b
PO-2	b	a	c	c (0.75)	b
				d (0.25)	
ALK-1	b	c	a (0.50)	c	a (0.25)
			c (0.50)		c (0.75)
ALK-2	a	b	a	b	a
ALK-3	a	—	a	—	a
ALK-4	b	b	b	a (0.35)	b
				b (0.65)	
ALK-5	—	a	b	c	a
EST-1	b (0.70)	a (0.40)	a (0.78)	a (0.42)	b (0.44)
	c (0.30)	b (0.60)	b (0.22)	b (0.58)	c (0.56)
EST-2	b	a	a	b	—
EST-3	a	a	a	a	b
EST-4	a (0.81)	a (0.71)	b (0.38)	a (0.53)	a (0.78)
	c (0.19)	c (0.29)	c (0.62)	b (0.47)	c (0.22)
LAP-1	a (0.37)	—	a (0.40)	—	—
	b (0.63)		b (0.60)		
LAP-2	a (0.50)	a (0.47)	a (0.50)	a (0.83)	b
	c (0.50)	c (0.53)	b (0.50)	c (0.17)	
LAP-3	a	—	—	a	a
LAP-4	b	a	b	b	b
LAP-5	a	a	a	a	a

Alleles are correspondingly lettered from "a". The value in parenthesis represents the frequency of each allele in population.
Aa=*Asterias amurensis*, *Aj*=*Aphelasterias japonica*, *Ca*=*Coscinasterias acutispina*, *Dn*=*Distolasterias nipon*, *Pb*=*Plazaster borealis*.

Table 2. Genetic variation in the five species of the family Asteriidae

Parameter	*Aa*	*Aj*	*Ca*	*Dn*	*Pb*
No. of alleles per locus	1.17	1.18	1.21	1.38	1.15
Proportion of polymorphic loci:P(%)	17.2	14.3	21.4	38.5	15.4
Expected average heterozygosity per locus:H(%)	7.6	6.4	8.5	16.7	5.9

Aa=Asterias amurensis, Aj=Aphelasterias japonica, Ca=Coscinasterias acutispina, Dn=Distolasterias nipon, Pb=Plazaster borealis.

Table 3. Genetic identities (above diagonal) and genetic distances (below diagonal) between five species of the family Asteriidae

Species	1	2	3	4	5
1. *Asterias amurensis*	—	0.475	0.434	0.484	0.598
2. *Aphelasterias japonica*	0.744	—	0.433	0.506	0.397
3. *Coscinasterias acutispina*	0.835	0.837	—	0.401	0.370
4. *Distolasterias nipon*	0.726	0.681	0.914	—	0.360
5. *Plazaster borealis*	0.514	0.924	0.994	1.022	—

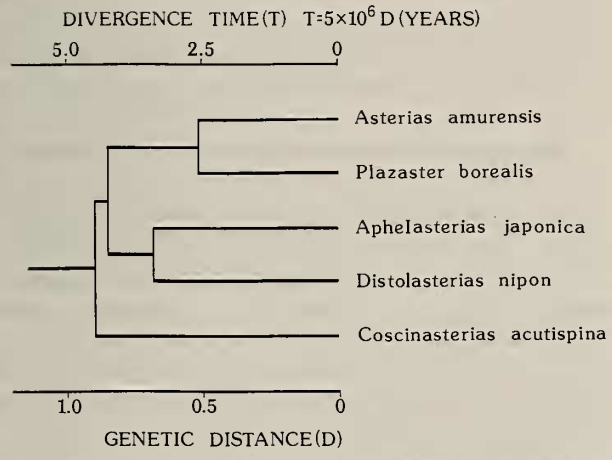

Fig. 3. A molecular phylogenetic tree for the five starfish species of the family Asteriidae. It was constructed from Nei's genetic distances by using the UPGMA clustering method of Sneath and Sokal [27]. The divergence time estimated from the Nei's equation [24] using the genetic distance is given in the phylogenetic tree.

species [14, 15, 19–21]. These results can be explained by the neutral mutation theory of Kimura [11]: The more strictly functional constraint would decrease the neutral regions of the molecules and the probability of a mutation change not being harmful (i.e.,selective neutral) is smaller for the substrate-specific enzymes than for nonspecific enzymes.

I have previously reported on the amount of genetic variation within populations of various echinoderm species [18]. It is interesting to compare the extent of genetic variation (the average heterozygosity per locus: the H value) in the five starfish species studied here with H values observed in other echinoderm populations. *A. amurensis, A. japonica, C. acutispina* and *P. borealis* showed low genetic variabilities (H=7.6, 6.4, 8.5, 5.9%) and these H values were comparable to those (H=0–8.7%) of many other shallow water echinoderms reported previously [18]. On the other hand, *D. nipon* showed much higher genetic variability (H=16.7%) than other shallow water echinoderms and the value was comparable to the H values of deep-sea echinoderms. Nei [25] and Nei and Graur [26] examined the relationship between average heterozygosity and population size for 77 different species. As a result, they found a significant correlation between them. From this evidence, it may be considered that the difference in the extent of genetic variation among five species is related to their population sizes. Namely, it may be expected that *D. nipon* showing the higher genetic variability has larger population size than the other four species. Further extensive population surveys in various marine invertebrates would be required for establishing the validity of this prediction.

Phylogenetic relationship of five species of the family Asteriidae

The molecular phylogenetic tree shown in Figure 3 clearly indicated that the five species of the family Asteriidae are phylogenetically divided into three large clusters: (i) *A. amurensis* and *P. borealis*; (ii) *A. japonica* and *D. nipon*; and (iii) *C. acutispina*. Fisher [6] suggested from the morphological standpoint that the family Asteriidae may be a large and polyphyletic aggregation of genera, and he proposed the subfamily system within the family Asteriidae. The heterogeneity of the family suggested by Fisher seems not contradictory to the present results, excluding the problems what species belongs to each subfamily.

The electrophoretic results showed that *A. amurensis* and *P. borealis* are the most closely related to each other among five species. The genetic distance (D=0.514) be-

tween them was comparable to the D values reported between congeneric species in other animals [29]. The close affinity between them was also suggested by the immunological study of Mochizuki and Hori [22]. They examined the phylogenetic relationships among various starfish species by using the enzyme inhibition method with the specific antibody against purified hexokinase (HK) from the pyloric ceaca of *A. amurensis*. The immunological data indicated that *P. borealis* has the highest immunological similarity to *A. amurensis* among seven species of the family Asteriidae examined. In addition, Fisher [6] stated from the morpholog-ical standpoint that *A. amurensis* and *A. japonica* may be closely related to *P. borealis*. His view is partially consistent with these biochemical results, excepting the phylogenetic position of *A. japonica*.

The allozymic study also showed the close affinity between *A. japonica* and *D. nipon*. The genetic distance (D = 0.681) between them was comparable to the D values observed between congeneric species or closely related confamilial genera in other animals [29]. Interestingly, there are two conflicting views on the systematic position of *A. japonica* from the morphological standpoint: Fisher [6] and Hayashi [8] proposed the close affinity between *A. japonica* and *A. amurensis*, and included these two species into the subfamily Asteriinae. On the other hand, Shigei and Saba (personal communication) suggested that *A. japonica* may be rather closely related to *D. nipon*. The present results are in favor of the view of Shigei and Saba. In contrast, the view of Fisher [6] and Hayashi [8] is inconsistent with not only the present electrophoretic study but also the immunological studies by other workers: Mochizuki and Hori [22] showed by the enzyme inhibition method that *A. japonica* is distantly related to *A. amurensis*. Prior to their study, Kubo [12] examined the phylogenetic relationships among various starfish species by the following immunological method: He prepared rabbit antisera against extracts of tube feet of ambulacral zones taken from several starfish species and measured the cross-reactivity of the antisera to antigens from various species by the quantitative precipitin technique. As a result, he obtained the similar results to those of Mochizuki and Hori [22]. However, there are some differences between the present electrophoretic data and Kubo's immunological results. Namely, Kubo [12] showed that *A. amurensis* was more distantly related to *A. japonica* than to *D. nipon* and *C. acutispina*. On the other hand, the present electrophoretic results (Fig. 3) indicated that *A. amurensis* and *P. borealis* are more closely related to the cluster of *A. japonica* and *D. nipon* than to *C. acutispina*. In spite of such differences, these biochemical studies did not support the close affinity between *A. japonica* and *A. amurensis* which was suggested by the morphological studies [6,8].

The molecular phylogenetic tree (Fig. 3) also indicated that *C. acutispina* is the most distant species of the five members. The result seems to be consistent with the zoogeographical evidence: Of the five species, *C. acutispina* is not commonly found in the cold seas of northern Japan and distributes widely in the more southern regions from central Honsyu to the Ryukyu Islands. On the other hand, the main distributional region of the other four species is the cold seas of northern Japan. From the morphological studies, Fisher [6] and Hayashi [8] proposed the close affinity between *C. acutispina* and *D. nipon*, and included these two species into the subfamily Coscinasterinae. However, their taxonomic system is inconsistent with the present electrophoretic results.

The molecular phylogenetic tree (Fig. 3) shows not only their genetic relationships, but also the sequence of their evolutionary divergence. According to Nei [24], genetic distance (D) corresponds well with the divergence time (T) from the common ancestor, and T of two taxa can be estimated by $T = 5 \times 10^6 D$ (years). Application to this equation to the molecular dendrogram constructed from the genetic distances leads to the following speculation of evolutionary process of the five species: Firstly, the common ancestor of the five species diverged into two lineages (one is *Coscinasterias* lineage and the other the common ancestor of the other four genera) 4.5 million years (MY) ago. Then, the latter ancestor diverged into two lineages (one is the *Asterias-Plazaster* lineage and the other the *Aphelasterias-Distolasterias* lineage) after a short time (4.3 MY ago). Finally, these four genera differentiated from one another 2.6–3.4 MY ago. The phylogenetic tree suggests that *Asterias* and *Plazaster* are more recent genera which evolved later.

The biochemical systematic studies of sea-urchins reported previously suggested that the more recent species which evolved later tend to become predominant species [14–17, 20]. Among the five species of the family Asteriidae, *A. amurensis* which evolved later seems to be more predominant species than others. The species is more frequently found in Japanese waters than the other four species and shows the extensive morphological variations between local Japanese populations in some morphological characters such as body color, body size, spine and so on. This may suggest the speciation within *A. amurensis*. At present, we have been investigating the genetic differentiation between local populations of *A. amurensis* by using enzyme electrophoresis and attempting the molecular approach concerning the speciation and evolution of the species.

As evident from Figure 1, *P. borealis* is considerably specialized at the morphological level, and shows the clear differentiation between disk and arm, in contrast with the other four species with standard morphology. The molecular phylogenetic tree (Fig. 3) implies that *P. borealis* of such specialized morphology might have differentiated from the *Asterias*-like starfish with standard morphology, since the cluster consisted of *P. borealis* and *A. amurensis* is also closely related to the cluster of *A. japonica* and *D. nipon* with standard morphology. If it is true, the evolutionary rate at the morphological level in the *Plazaster* lineage might have been much accelerated. In future, further detailed investigation on the close genetic relationship between *P. borealis* and *A. amurensis* which highly differentiated with each other at the morphological level would produce some useful and

valuable information on the morphological evolution in starfish.

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